Alterations in the Metabolism of Filaggrin in the Skin After Chemical- and Ultraviolet-Induced Erythema

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We have investigated the effect on the normal synthesis and metabolism of filaggrin of treatment of guinea pig skin with a chemical irritant, hexadecane, or with erythemal doses of UV radiation.

Examination of the skin by immunofluorescence with an antiserum against filaggrin demonstrates 3 phases of the response. The first phase is an apparent stabilization of the filaggrin present at the time of treatment. Thus, a zone of stratum corneum is produced which moves up toward the skin surface over the days following treatment, without the loss of immunoreactivity which normally results from the metabolism of filaggrin to free amino acids.

The second phase of the reaction, which occurs during the first day after treatment, is a loss of immunoreactive material from the upper viable epidermis, which results over the next day in the formation of a zone of filaggrin-deficient stratum corneum. The third phase, 2–3 days after the treatment, is the reestablishment of immunoreactivity in the newly re-formed granular layer, followed by the formation of an immunoreactive zone at the bottom of the stratum corneum. This zone remains very thin despite the rapid passage of cells through it. This shows that the filaggrin being formed during this phase of the reaction is being broken down normally as the stratum corneum matures.

Investigations of the kinetics of filaggrin synthesis and breakdown using a [^{3}H]histidine pulse/chase method, confirm the impression gained from immunofluorescence studies that the time between formation and breakdown of the filaggrin is much reduced in the hyperplastic epidermis resulting from the irritation. Thus, although the hyperplasia is reflected in a thickening of malpighian and granular layers of the epidermis, it does not result in any thickening of the filaggrin-positive zone at the bottom of the stratum corneum. This suggests the action of a control mechanism designed to prevent the extension of this filaggrin-positive zone into the upper stratum corneum. *J Invest Dermatol 87:460–465, 1986*

Keratohyaline granules are one of the most prominent ultrastructural features of normal epidermis. They occupy a substantial fraction of the total cytoplasmic volume of the epidermal cell in the final stage of its differentiation prior to the formation of the flattened squame of the stratum corneum. The importance of these granules in the formation of a normal stratum corneum is emphasized by the strikingly different properties of the stratum corneum produced in their absence, for example the hard inflexible scales of the mouse tail, the loose flaky scales of severe psoriasis, or the scaling and cracking stratum corneum produced by severe irritation.

The main protein component of the keratohyaline granule has only recently been isolated in an undegraded form [1]. It is an unusually large protein ($M_r > 300,000$) with a characteristic amino acid composition containing a large fraction of basic amino acids. The protein itself has, however, a neutral isoelectric point in all species studied [2] due to extensive phosphorylation of serine residues in the protein. Reports of similar but smaller and of large but heterogeneous proteins isolated from mouse or rat keratohyaline [3–5] reflect the extreme sensitivity of the protein to proteases present in epidermal extracts [1,6].

Two functions have been proposed for this protein. Dale et al [7] isolated a protein from stratum corneum which was derived from the keratohyaline granule protein by dephosphorylation and proteolysis. This protein had a very basic isoelectric point and had the property of binding together keratin fibers into large bundles reminiscent of the close packed "keratin pattern" seen at high magnification in normal stratum corneum [8]. They suggested therefore that the function of the protein was essentially structural and proposed the name filaggrin (*filament aggregating protein*) to reflect this. However, recent work has cast doubt on the in vivo relevance of this keratin aggregating ability as, in ichthyosis vulgaris, a complete absence of filaggrin appears to have no effect on the normal pattern of keratin aggregation [9]. Nonetheless, this terminology will be used henceforth in this paper, with filaggrin referring to the basic forms of the protein found in the stratum corneum, and profilaggrin referring to the neutral high-molecular-weight phosphorylated form found in the keratohyaline granule.

The second function proposed for the protein was suggested by our observation that the dephosphorylated filaggrins had short lifetimes in vivo and were degraded completely to free amino acids well before the cells containing them were shed from the skin [10]. This finding has since been independently confirmed [11]. Proteolysis of filaggrin proved to be the major, or even the only source of a pool of water-soluble materials found in high

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Abbreviations:
DTT: dithiothreitol
FITC: fluorescein isothiocyanate
MED: minimal erythemal dose
PBS: phosphate-buffered saline
PMSF: phenylmethylsulfonyl fluoride
SDS: sodium dodecyl sulfate

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concentrations in healthy stratum corneum. These include urocanic acid [12] and pyrrolidone carboxylic acid [13] which have been proposed to act, respectively, as an absorber of harmful UV radiation [14] and a hygroscopic substance allowing the stratum corneum to remain hydrated and hence flexible in low environmental humidity [15].

The loss of this second function in epidermis failing to produce keratohyaline granules would account in part for the properties of dryness and inflexibility commonly seen in stratum corneum produced by such an epidermis. Indeed it has been observed that in the psoriatic stratum corneum the levels of soluble water-binding materials are very low (for review see [16]), which is consistent with the absence of keratohyaline granules in this disease.

Since alterations in the production and metabolism of filaggrin can clearly have marked effects on stratum corneum structure and function, the present work was carried out in order to investigate in greater detail the alterations that may take place during relatively mild perturbation of normal epidermis by application of the chemical irritant hexadecane and by UV irradiation, both of which result in flaking of the skin surface.

MATERIALS AND METHODS

Treatment of Animals Guinea pigs were of the Dunkin-Hartley (Colworth) strain. Males weighing approximately 400 g were clipped using small animal clippers immediately before treatment with hexadecane, or shaved with an electric shaver before irradiation. Hexadecane was applied once only at a dose of 25 μl/cm^2 to the lateral skin. UV irradiation was by a water-cooled medium-pressure mercury vapor arc (Hanovia Kromayer Model 10) with a Schott WG305/3 filter. Intensity was measured with a spectroradiometer and converted to absolute terms by chemical actinometry. The intensity of radiation at different wavelengths is given in Table I.

Preparation of Guinea Pig Filaggrin Guinea pig filaggrin was prepared by a modification of our published procedure [2]. Briefly, epidermis was extracted in 1.0 M sodium phosphate pH 7 containing protease inhibitors EDTA and phenylmethylsulfonyl fluoride (PMSF) at 2 mM. The solubilized proteins were precipitated by dialysis against water, dissolved in 8 M urea buffer, and chromatographed on DE52 cellulose. Final purification was achieved by the formation of macrofibrils between the filaggrin and purified keratin fibers as previously described [2] followed by solubilization of the macrofibrils in 8 M urea, 10 mM Tris/HCl pH 8, 10 mM dithiothreitol, and absorption of the keratin on DE52 cellulose. The pure filaggrin was dialyzed against 0.1% sodium dodecyl sulfate (SDS) and stored frozen.

Preparation of Antifilaggrin Antiserum Rabbits were immunized with the purified guinea pig filaggrin in Freund's complete adjuvant and boosted at 14-day intervals with the same material. The antiserum was affinity-purified by passage through a small column of guinea pig filaggrin bound to cyanogen bromide-activated Sepharose 4B. The bound antibody was washed with phosphate-buffered saline (PBS) containing 0.5 M NaCl and eluted with 4 M $MgCl_2$. The eluted antibody was rapidly desalted into PBS and used at a final dilution of 1:50 (relative to the original serum). Preimmune serum was treated in the same way.

The affinity-purified antiserum was characterized by staining a nitrocellulose blot of a 2-dimensional polyacrylamide gel of urea/dithiothreitol (DTT)-soluble epidermal proteins prepared as described previously [2]. The proteins were electrophoretically blotted and the blot stained using a 1:200 dilution of the antiserum as described in the Bio-Rad Transblot Cell protocol. The result is shown in Fig 1. Guinea pig filaggrin and profilaggrin are a complex family. The stained spots shown in Fig 1 all correspond to filaggrin species identified by [^{3}H]histidine incorporation and specific interaction with keratin filaments as shown previously [2].

Microscopy Pieces of skin were fixed for 2 h at 0°C in 3.5% freshly hydrolyzed paraformaldehyde in PBS and washed overnight at 4°C against several changes of 15% sucrose in PBS. They were immersed in Tissue-Tek OCT compound and rapidly frozen in isopentane/liquid nitrogen prior to cryostat sectioning at 4 or 8 μm.

Sections were air dried, moistened with PBS (10 min), overlaid with 50% fetal calf serum (10 min), reacted with antifilaggrin serum diluted in 50% fetal calf serum (30 min), washed in PBS (3 × 10 min), reacted with fluorescein isothiocyanate (FITC)-conjugated goat antirabbit serum (1:50 in 50% fetal calf serum) washed in PBS (3 × 10 min), and mounted in glycerol/paraphenylene diamine medium [18]. The FITC-conjugated serum was diluted to × 25 in PBS and absorbed at a ratio of 100 mg/ml with a preparation of 1 M phosphate buffer pH 7 insoluble epidermal protein prior to use. This reduced the nonspecific staining of the stratum corneum which otherwise interfered with the specific staining.

The following controls were carried out. Preimmune rabbit serum gave diffuse cytoplasmic fluorescence in epidermal cells and diffuse fluorescence throughout the stratum corneum. Affinity purification of the preimmune serum eliminated this fluorescence which was probably due to the antikeratin autoantibodies often found in rabbit serum. The combination of affinity-purified preimmune serum and absorbed FITC-conjugated serum gave very little fluorescence—which was not visible in photographs exposed for the same time as those shown in this paper. Sections stained with hematoxylin and eosin were prepared from blocks, fixed with Bouin's fixative, and processed through paraffin by standard methods.

Interconversion of [^{3}H]Profilaggrin, Filaggrin, and Free Amino Acids To measure the effect of hexadecane treatment on the metabolism of preformed profilaggrin and filaggrin, guinea pigs were injected intradermally at 3 sites on each side with 40 μl

Table I. Ultraviolet Radiation Protocol

Wavelength (nm)	Intensity (mW cm^{-2})	Erythemal Power[a] (mW cm^{-2} = 297 nm)
289	0.064	0.016
292	0.067	0.047
297	1.287	1.287
302	4.861	2.674
313	15.8	0.474
365	28.6	0.00

[a]This is the intensity in mW cm^{-2} multiplied by the relative effectiveness of that wavelength in inducing a 24-h erythema in human skin, relative to the effectiveness of a wavelength of 297 nm [17].

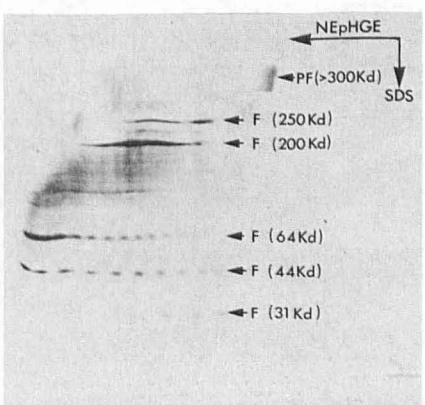

Figure 1. Immunoblot of an extract of 8 M urea/DTT soluble epidermal proteins against affinity-purified antifilaggrin antiserum. *PF* = Profilaggrin; *F* = filaggrin.

of L-[2,5-^{3}H]histidine (40 Ci/mmol, 1 mCi/ml). After either 6 h, when the radioactive label was principally present in profilaggrin [1], or 48 h, when the radioactive label had passed into filaggrin [1], one side of the animal was treated with hexadecane as described above. Animals were taken at intervals after this treatment, the epidermis isolated from the immediate site of the injection by freeze-scraping, and the perchloric acid-soluble radioactivity measured as described previously [12].

The kinetics of interconversion of profilaggrin, filaggrin, and free amino acids during the recovery phase after hexadecane treatment were investigated by injecting guinea pigs with [^{3}H]histidine, as described above, 4 days after treatment with hexadecane. Samples of epidermis were taken at intervals by freeze-scraping and extracted either in 2% SDS, 0.1 M Tris/HCl pH 8, 2 mM EDTA, 2 mM PMSF, or in cold 0.4 M perchloric acid. The insoluble material was dissolved in Soluene 350 (Packard) and counted. The SDS extract was analyzed by SDS polyacrylamide gel electrophoresis and the profilaggrin band cut out, dissolved, and counted [1]. The radioactivity in profilaggrin and the perchloric acid-soluble fraction was expressed as a percentage of the total epidermal radioactivity, i.e., perchloric acid-soluble plus acid-insoluble counts, or SDS/DTT-soluble plus insoluble counts.

Profilaggrin is an exceptionally labile protein and, to be confident that the methodology described accurately measures the total labeled protein, it is necessary that proteolytic action on the profilaggrin be prevented. Of the various methods of epidermal isolation and extraction available, freeze-scraping followed by immediate homogenization in 2% SDS plus protease inhibitors is the least likely to allow proteolysis to take place. Using this method, profilaggrin isolation without detectable proteolysis has been demonstrated [1] and independently confirmed [6].

RESULTS AND DISCUSSION

Filaggrin Distribution in Normal Skin A section of normal guinea pig dorsal skin stained with an antibody to filaggrin is shown in Fig 3*a*. This antibody binds to profilaggrin and the whole complex filaggrin family on immunoblots and therefore indentifies the location of the protein from its precursor form in the keratohyaline granule to the small peptides formed just prior to its final proteolytic destruction [1,2] (Fig 1).

In normal skin, the granular layer is so thin that the fluorescence due to keratohyaline granules can barely be distinguished from that due to the stratum corneum. It can be seen clearly, however, that filaggrin is present only in the lowermost layers of the stratum corneum. The complete absence of specific fluorescence from the upper layers confirms our biochemical evidence that the filaggrin is completely degraded early in the maturation of the stratum corneum [10].

Changes in Filaggrin Distribution During the Response of Skin to Hexadecane Figures 2 and 3 show sections of guinea pig skin 1, 2, 3, and 4 days after a single application of hexadecane, stained with hematoxylin and eosin (Fig 2) or by immunofluorescence with antifilaggrin serum (Fig 3). At day 1, the granular layer has disappeared and a thin zone of new stratum corneum which appears darker by phase contrast than normal stratum corneum has appeared. This new stratum corneum does not appear to contain filaggrin and is therefore presumably formed by the premature differentiation of epidermal spinous cells that have failed to go through a granular phase. Above this zone of new stratum corneum is a band of stratum corneum which does contain filaggrin. This appears to be derived either from the granular cells already in existence at the time of the hexadecane treatment,

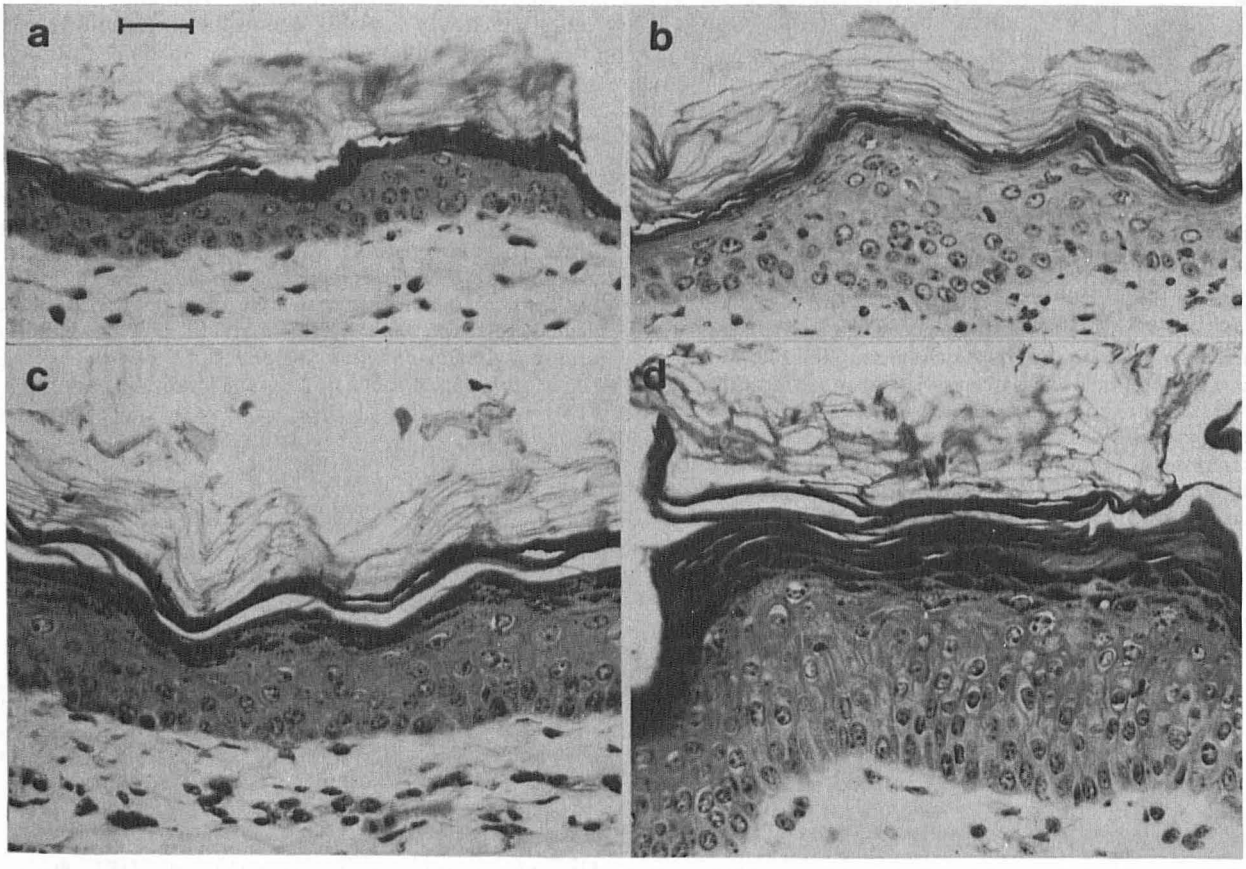

Figure 2. Histologic changes in guinea pig epidermis after hexadecane treatment. *a*, Normal skin. *b–d*, Skin 1, 2, and 3 days after a single application of hexadecane. *Bar* = 30 μm.

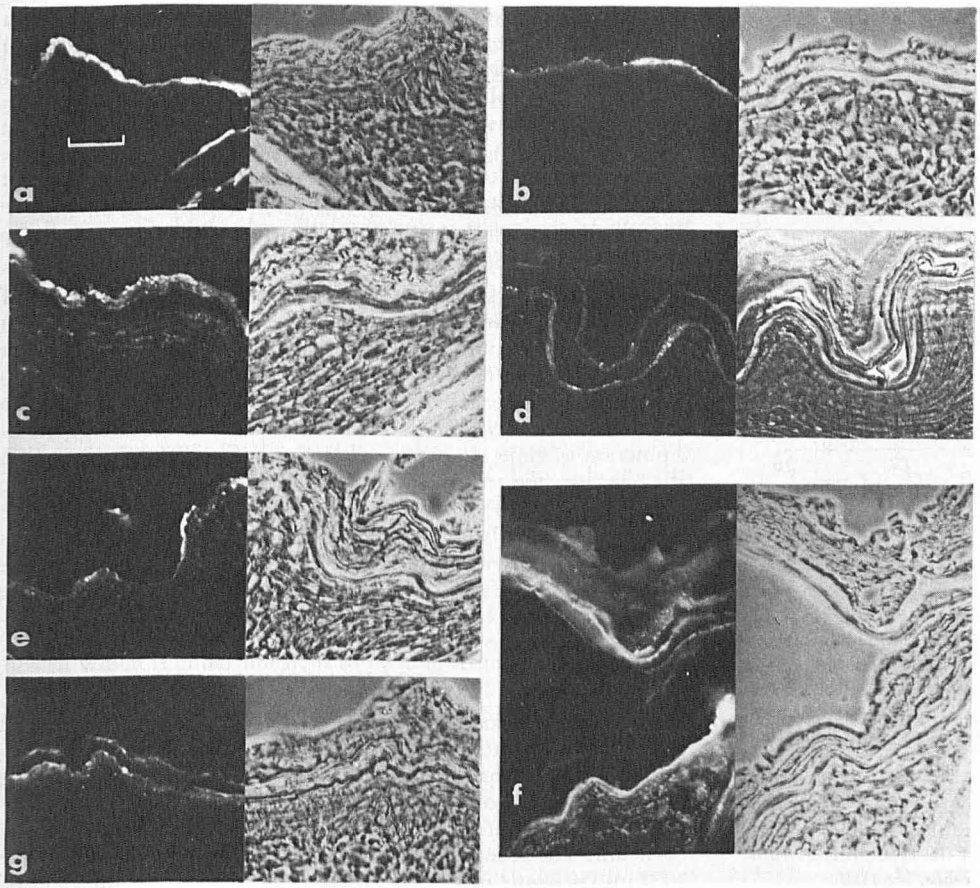

Figure 3. Changes in filaggrin distribution determined by indirect immunofluorescence during the irritant response. *a*, Normal skin. *b–e*, Skin 1, 2, 3, and 4 days after a single application of hexadecane. *f*, Area of skin where flaking is in progress 4 days after hexadecane treatment. *g*, Skin 3 days after exposure to 2 MEDs of UV radiation. In all figures except (*f*) the image on the right is a phase contrast micrograph of the same section shown by immunofluorescence on the left, but reversed to present a mirror image of the immunofluorescent micrograph. *Bar* = 30 μm.

or from the preexisting zone of filaggrin-positive stratum corneum.

At day 2 after the hexadecane treatment, the band of new, filaggrin-free stratum corneum has thickened but the granular layer has reestablished itself. No new filaggrin-positive stratum corneum has yet been produced but the old filaggrin-positive zone of the stratum corneum is still evident. This represents a further pertubation of normal stratum corneum metabolism as this filaggrin should at this stage have been proteolytically destroyed. There are thus 2 distinct defective zones in the stratum corneum, one in which no filaggrin has been produced, overlaid by a zone where the normal proteolysis of filaggrin has been prevented.

It is always necessary to interpret negative immunofluoresence results with caution, particularly as antigenic masking of filaggrin in skin has been demonstrated [19]. However, the 2 filaggrin-negative zones of the stratum corneum described above are unlikely to result from artifactual masking. The absence of filaggrin from the normal superficial stratum corneum has been confirmed biochemically [10]. The absence of filaggrin from the defective stratum corneum produced after the disappearance of the granular layer is entirely consistent with the known localization of the filaggrin precursor in the keratohyaline granules.

By day 3 after the treatment (Fig 3*d*), the newly formed granular layer has produced a normal-appearing zone of filaggrin-positive stratum corneum underlying the 2 defective zones. The constant thickness of the zone seen a day later (Fig 3*e*) is consistent with the proteolysis of the filaggrin in this zone being normal.

It is at this stage that flaking of the skin surface takes place and the plane of cleavage typically appears in the zone of filaggrin-negative stratum corneum produced during the initial injury reaction (Fig 3*f*).

By day 7 the stratum corneum appears normal again although the living cell layers are still hyperplastic. The fact that the thickness of the filaggrin-positive zone of the stratum corneum is very similar to that seen in untreated skin is of interest. The rate of cell turnover in hyperplastic skin is more rapid than in normal skin [20]. Thus, to maintain a constant thickness of filaggrin-positive stratum corneum, the catabolism of the filaggrin, once formed from the profilaggrin, must be more rapid. In contrast, the kinetics of formation and dephosphorylation of the profilaggrin in the keratohyaline granules need not necessarily be accelerated as the granular layer increases in thickness in response to the increased speed of cell turnover.

This increase in rate of catabolism of filaggrin is confirmed by biochemical studies of the rate with which an injected radioactive amino acid passes through the protein pool and appears as free amino acid retained within the stratum corneum. Figure 4 shows a comparison of the kinetics of this process in normal and hyperplastic skin. The lifetime of the profilaggrin is reduced from a mean of approximately 20 h to 6 h. The corresponding filaggrin lifetime is drastically shortened from 70 h to 9 h. It appears therefore that a mechanism exists whereby the epidermis can regulate the rate of proteolysis of the filaggrin, in order to maintain at a constant thickness the layer of stratum corneum in which undegraded filaggrin exists.

One detail concerning the data in Fig 4 should be clarified. It appears that profilaggrin accounts for 40% of the total epidermal radioactivity shortly after injection of the isotope, while the free amino acids derived from it account for 60% of the total a few days after the injection. The reason for this discrepancy lies partly in a reduction of the total epidermal radioactivity as proteins in the living cells are metabolized, and partly in a continued slow synthesis of radioactive profilaggrin for several hours after the injection of radioactive amino acid, i.e., the experiment is not a pure pulse-chase situation. Details of the experimental data supporting this explanation of the discrepancy are given by Scott et al [10].

A further point raised by the immunofluorescence study which

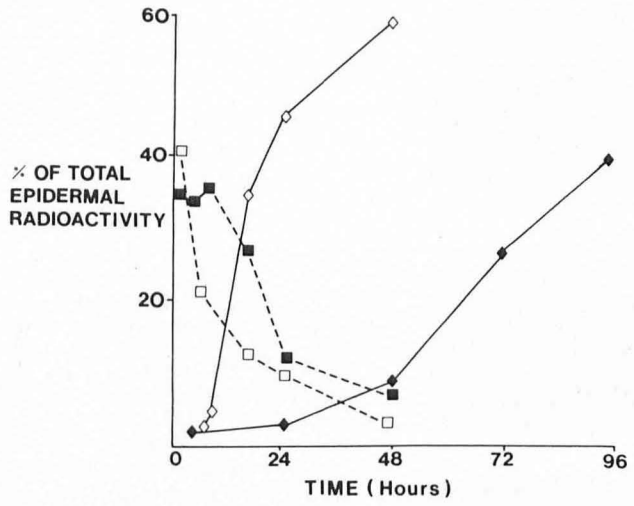

Figure 4. Rate of interconversion of profilaggrin, filaggrin, and amino acids during the hyperplastic response. The percentage of total epidermal radioactivity from a pulse of [^{3}H]histidine in profilaggrin (*squares*) and free amino acids (*diamonds*) was measured as a function of time in normal skin (*solid symbols*) and skin rendered hyperplastic by treatment with hexadecane (*open symbols*) 4 days prior to injection of the [^{3}H]histidine. The mean lifetime of profilaggrin was taken as the time required for the percent of radioactivity in profilaggrin to fall from 40% to 20%. The mean lifetime of filaggrin was taken as the difference between this figure and the time for the level of ^{3}H-labeled free amino acids to reach half its maximum level, i.e., 30%. The reason for profilaggrin accounting for only 40% of the total epidermal radioactivity at early times while the free amino acids derived from it at later times account for 60% of the total radioactivity is related to the loss of some radioactivity from the epidermis as some cellular proteins are turned over (for a full discussion see [10]).

was clarified by biochemical means was the extent and significance of the failure of the stratum corneum to degrade the filaggrin or profilaggrin already present at the time of the hexadecane treatment. The presence of a zone of apparently undegraded filaggrin in immunofluorescent images indicates only the presence of some filaggrin and gives no reliable indication of the quantities involved. To measure the amount of this undegraded filaggrin the epidermis was prelabeled either 6 h or 48 h prior to hexadecane treatment, so that either the profilaggrin pool or the filaggrin pool was labeled at the time of the hexadecane application. Any failure to catabolize the filaggrin normally would then be indicated by a reduced eventual appearance of radioactivity in the free amino acid pool, as filaggrin is normally completely degraded to free amino acids [10]. The results of this experiment are shown in Fig 5. No significant reduction in the catabolism of either profilaggrin or filaggrin was evident. It therefore follows that either: (1) the residual undegraded filaggrin detected by immunofluorescence is only a small fraction of the whole; or (2) the filaggrin is degraded to species that are perchloric acid soluble but still antigenically reactive. In normal skin, the acid-soluble pool of ^{3}H-labeled material present 4 days after injection of the radioisotope can be identified by isotope dilution analysis as being entirely free histidine and urocanic acid [12] even though there is a considerable quantity of small peptide fragments of filaggrin present at this time [10]. It is improbable therefore that the residual immunofluorescent stain is due to small, acid-soluble filaggrin fragments—although the possibility cannot be completely excluded.

Changes in Filaggrin Distribution During the Response of Skin to UV Radiation Guinea pigs were UV irradiated with 1, 2, and 3 minimum erythemal doses (MEDs), defined by that dose giving slight patchy erythema at 24 h after irradiation. The clearest response was produced at 2 or 3 MEDs; at 1 MED the response was similar but patchy. The distribution of filaggrin as a function of time after irradiation was very similar to that seen after hexadecane treatment. Flaking of the skin occurred at the same time as occurred with hexadecane and the plane of cleavage of the flakes was also the same. Fig 3*g* shows the presence, 3 days after irradiation with 2 MEDs of UV radiation, of both a zone of residual, undegraded, filaggrin and a zone of filaggrin-negative stratum corneum comparable to that seen at 3 days after hexadecane treatment.

CONCLUSIONS

Premature keratinization of epidermal cells that have not yet passed through the granular stage appears to be a common reaction to the 2 different damaging stimuli used. This study emphasizes the serious consequences of this failure to complete the terminal differentiation program on the subsequent makeup of the stratum corneum. The absence of filaggrin from a substantial zone of the stratum corneum might be expected to have 2 possible effects. The first might be to alter the keratin packing in the squames [7], although the observation of Sybert et al [9] that absence of filaggrin has no effect on the keratin pattern of ichthyotic stratum corneum casts doubt on this possibility. The second effect of loss of filaggrin would be to reduce the ability of the squames to remain hydrated as they move up through the stratum corneum [8].

It would seem possible that this latter effect contributes markedly to the skin flaking subsequently seen. First, the plane of cleavage of the stratum corneum runs through the filaggrin-deficient zone. Second, the cleavage occurs at approximately the same time as the first layers of "normal" stratum corneum are laid down below the layers of defective cells. Since the defective cells are unlikely to provide a fully effective barrier to the outward diffusion of water, the production of the first layers of "normal" stratum corneum might mark the first occasion that the defective stratum corneum is exposed to low water activity. It would be at this time, therefore, that the lack of water-binding substances would be felt and the defective stratum corneum would become

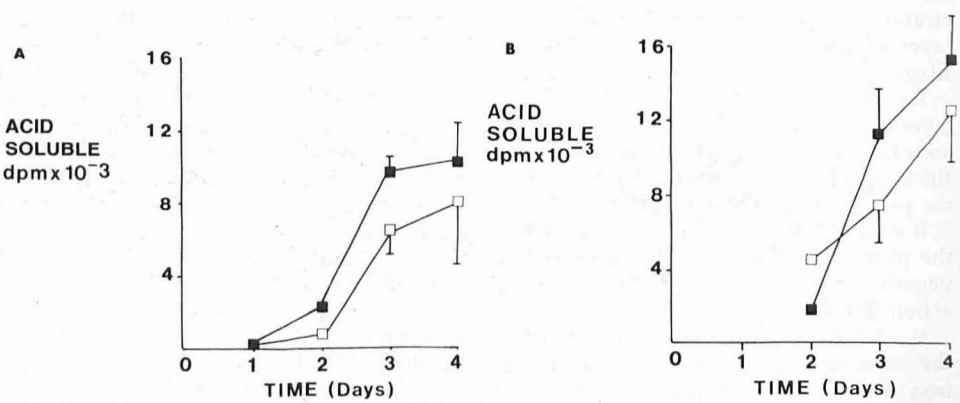

Figure 5. Effect of hexadecane treatment on the conversion of preformed profilaggrin and filaggrin to amino acids. Guinea pigs were pulse-labeled with [^{3}H]histidine at zero time and treated on one side (*open square*) with hexadecane 6 h (*A*) or 24 h (*B*) after the labeling. The rate and extent of appearance of radioactivity in the free amino acid pool was followed as a function of time and compared with the untreated control side of the same animal (*solid square*). Each point shows the mean and SD of 6 measurements on 2 animals.

dehydrated and inflexible, which could lead to failure to accommodate to stress and hence cracking and flaking of the structure. This effect should not of course be seen in isolation. Defective terminal differentiation would have other effects than on filaggrin synthesis, such as altered intracellular lipids and desmosomes which might also contribute to the failure of the stratum corneum in this defective zone.

The demonstration that filaggrin is restricted to a thin zone at the bottom of the stratum corneum confirms our earlier biochemical data which indicated that the lifetime of the filaggrin in the stratum corneum is only a fraction of the turnover time of the stratum corneum [10]. As yet, there is no information available relating to the nature of, or control of, the proteolytic enzymes responsible for the filaggrin degradation. In the present study we have demonstrated that filaggrin degradation can be accelerated at least 8-fold in order to maintain the narrowness of the filaggrin zone in the stratum corneum despite the greatly accelerated rate of cell turnover in hyperplasia. This indicates that the proteolytic reactions are controlled in some way such that they are activated as soon as the cell reaches a critical point in its movement through the stratum corneum toward the skin surface.

The logic behind this control is relatively simple. The stratum corneum acts as an effective barrier to water passage. There is, therefore, a gradient of water activity through the stratum corneum which is either linear, if the whole structure has a uniform resistance to water movement, or curved, in a manner dependent on one's assumptions about alterations in diffusion constant with position in the stratum corneum [21]. In either case, at some point in the structure the water activity will reach a point where a large reduction in the free water content of the cell would occur. The generation at this point of a large quantity of hygroscopic low-molecular-weight substances would enable the cell to remain hydrated beyond this point. Since the point at which the hygroscopic materials would be required would be dictated by the geometry of the stratum corneum rather than by the age of a particular cell within it, a mechanism controlling the proteolysis of the filaggrin protein should not be kinetically controlled but rather, should be controlled by some influence dependent on the particular conditions to which a cell is exposed within the stratum corneum.

We have evidence that the signal that turns on the proteolytic breakdown of filaggrin is, in fact, the gradient of water activity existing across the stratum corneum [22]. This control mechanism would ensure that filaggrin breakdown occurs precisely where the water-binding substances derived from filaggrin become necessary for the maintenance of stratum corneum hydration.

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